EXHIBIT Q

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Pharmacokinetics of *N*-Nitrosodimethylamine in Beagles[1]

Charles T. Gombar,[2] Harry M. Pylypiw, Jr., and George W. Harrington

Department of Drug Metabolism, Smith Kline & French Laboratories, Swedeland, Pennsylvania 19479 [C. T. G.], and Department of Chemistry, Temple University, Philadelphia, Pennsylvania 19122 [H. M. P., G. W. H.]

ABSTRACT

The pharmacokinetics of *N*-nitrosodimethylamine (NDMA) has been studied in beagles. Four male beagles were given 0.5- and 1.0-mg/kg doses of NDMA i.v. and 1.0- and 5.0-mg/kg doses p.o., and at appropriate times after dosing blood samples were drawn and the concentration of NDMA was measured. The experiments were separated by at least 1 week. Following a bolus i.v. dose, the concentration of NDMA in blood declined biphasically with a mean distribution half-life of 19 min and a mean elimination half-life of 73 min. The areas under the blood concentration *versus* time curves were proportional to the dose indicating that the pharmacokinetics in this dose range were first order. The mean systemic clearance was 43.3 ml/min/kg, the volume of distribution at steady state was 1.9 liters/kg and the mean residence time was 45 min. The clearance of NDMA in the dog was entirely metabolic because no NDMA could be detected in urine after i.v. dosing. The areas under the curve and maximum concentration in blood after the two p.o. doses were not proportional to dose. The evidence suggests that the pharmacokinetics of the 1.0-mg/kg dose were first order, but at the 5.0-mg/kg dose the metabolism of NDMA was saturated. The bioavailability of the lower p.o. dose (*i.e.*, the fraction of the dose that reached the systemic circulation) averaged 93%. The high bioavailability was unexpected since, in the rat, the bioavailability of NDMA is only about 10%, and the systemic clearance in the dog exceeds hepatic blood flow. These data suggest that a substantial fraction of the systemic clearance is extrahepatic and that the pharmacokinetics of NDMA in higher species may be quite different from that observed in rodents.

INTRODUCTION

The carcinogenicity of NDMA[3] and related nitrosamines has been demonstrated in at least 39 species (1, 2), but there is no direct evidence linking nitrosamines to human cancer. The mechanism of the carcinogenicity of NDMA is generally believed to involve alkylation of DNA, and the alkylating moiety results from metabolic activation of NDMA. Indirect evidence, such as comparative *in vitro* metabolism using tissue slices (3), homogenates (4), or explant cultures (5–10), suggests that the metabolic pathway responsible for generating the ultimate carcinogen is operative in humans. Furthermore, DNA from humans poisoned with NDMA was shown to contain the methylated bases 7-methylguanine and O^6-methylguanine (11).

Extrapolation of carcinogenicity data from animals to humans is fraught with difficulty. The inherent susceptibility of tissues to the carcinogenic action of NDMA, the efficiency and fidelity of repair processes, quantitative and qualitative metabolic aspects, and the pharmacokinetics of the compound may be very different in humans. Some of these problems can be studied in isolation. For instance, the availability of a suitable data base may allow extrapolation of the pharmacokinetic data from animals to humans. Currently, the only species for which good pharmacokinetic data exist are the rat (12) and the rabbit (13). The pharmacokinetics of NDMA and deuterated NDMA following low doses to the rat was studied by Mico *et al.* (12). Other information on the pharmacokinetics in rats comes from a study by Skipper *et al.* who developed a pharmacokinetic model for NDMA based on *in vitro* data (14) and other studies in which pharmacokinetic information was obtained at very high doses, or indirectly through measurement of exhaled $^{14}CO_2$ or DNA alkylation after administration of radiolabeled NDMA (15–20).

These studies have shown that NDMA, when administered p.o. at low doses, is well absorbed from the gastrointestinal tract, but only a very small fraction, about 10%, of the dose passes through the liver into the general circulation. This was shown directly by measuring the concentration of NDMA in blood after p.o. or i.v. administration (12) and indirectly by measuring the extent of DNA alkylation in kidney relative to liver after p.o. and i.v. dosing (18–20). The extent of first pass metabolism in species other than the rat is not known.

Interspecies scaling of pharmacokinetic data is difficult, especially when the compound is cleared primarily by metabolism, and extrapolation is virtually impossible when data are available for only one or two species. If several species are studied, allometric analysis may allow a reasonable interspecies extrapolation (21–27).

The purpose of the present study, and others in progress, is to collect detailed information on the pharmacokinetics of NDMA in mammalian species other than the rat to eventually allow interspecies comparisons to be made and, if possible, to extrapolate the data to describe the pharmacokinetics of NDMA in humans.

MATERIALS AND METHODS

Chemicals. *N*-Nitrosodimethylamine was purchased from Sigma Chemical Co. (St. Louis, MO). *N*-Nitrosodi[*methyl*-^{14}C]amine was synthesized from di[*methyl*-^{14}C]amine, purchased from Amersham Corp. (Arlington Heights, IL), by the method of Dutton and Heath (28). Morpholine (Aldrich Chemical Co., Milwaukee, WI) was double distilled, and the second distillate was collected and stored under nitrogen gas. Antifoam B was purchased from Fisher Scientific Co. (King of Prussia, PA). All other chemicals and solvents were ACS reagent grade or better.

Animals and Treatments. Four male purebred beagles obtained from Marshall Farms USA, Inc. (North Rose, NY), were used in this study. The dogs were placed in free standing slings and catheters were placed in the right and left saphenous veins. For i.v. dosing, NDMA dissolved in 0.9% NaCl solution was administered as a bolus dose in a volume of 0.2 ml/kg in one leg, and at appropriate times after dosing 5-ml blood samples were drawn into heparinized syringes from the catheter in the contralateral leg. Blood samples were immediately frozen in an acetone-dry ice bath and stored at −80°C until analyzed. Dosing experiments p.o. were performed in a similar manner except that the NDMA, dissolved in water, was administered in gelatin capsules. After blood sampling was completed, the dogs were housed in stainless steel metabolism cages and urine was collected for 24 h. Immediately prior to dosing and 24 h after dosing, serum samples were obtained for analysis

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[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: NDMA, *N*-nitrosodimethylamine; AUC, area under the blood concentration *versus* time curve from time zero to infinity; Cl_s, systemic clearance from blood; $Cl_{p.o.}$, oral clearance; Cl_{int}, intrinsic hepatic clearance; V_{ss}, volume of distribution at steady state; MRT, mean residence time; C_{max}, maximum concentration in blood; t_{max}, time to maximum concentration; $t_{1/2}$, apparent elimination half-life following oral administration; $t_{1/2}\alpha$, half-life of the first disposition phase following i.v. administration; $t_{1/2}\beta$, half-life of the second disposition phase following i.v. administration.

of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and γ-glutamyl transferase activities, total protein concentration, and the albumin/globulin ratio.

Determination of NDMA in Blood and Urine. The concentration of NDMA in blood and urine was determined as described by Pylypiw *et al.* (29). Briefly, 2.0 ml of blood or 5.0 ml of urine were simultaneously distilled and extracted into methylene chloride using a specially designed distillation-extraction apparatus. Following concentration of the methylene chloride extract, NDMA was determined by gas chromatography-thermal energy analysis using a thermal energy analyzer, Model TEA-502 (Thermo-Electron Corp., Waltham, MA), interfaced with a Hewlett Packard Model 5791A packed column gas chromatograph (Avondale, PA).

Plasma Protein Binding. The binding of [^{14}C]NDMA to plasma protein was measured in fresh heparinized dog plasma over an initial concentration range of 1 to 1000 ng/ml using the Centrifree micropartition system (Amicon Corp., Danvers, MA). Preliminary experiments demonstrated that [^{14}C]NDMA did not adhere to the filtration device.

Pharmacokinetic Calculations. The blood concentration *versus* time data following i.v. administration were fit to a two compartment open model using the PHARM program of Gomeni (30). Fitting was done either by curve peeling of the log transformed data or by weighted ($1/y$) nonlinear regression. The goodness of the fit was assessed by visual inspection of the fit and the residual plot. Noncompartmental methods were used to calculate Cl_s, V_{ss}, and MRT (31). The area under the blood concentration *versus* time curve from time zero to the last data point was calculated using the trapezoidal rule. The area from the last data point to infinity was estimated by dividing the concentration at the last time point by the apparent elimination rate constant.

The absorption half-life was calculated using Equation A where MAT, the mean absorption time, is the difference between the MRT after p.o. administration and the MRT after i.v. administration.

$$\text{Absorption } t_{½} = 0.693 * \text{MAT} \quad \text{(A)}$$

The apparent oral bioavailability (F) was determined using Equation B.

$$F = \frac{\text{AUC}_{\text{p.o.}} * \text{dose}_{\text{i.v.}}}{\text{AUC}_{\text{i.v.}} * \text{dose}_{\text{p.o.}}} \quad \text{(B)}$$

$Cl_{\text{p.o.}}$ is the dose divided by $\text{AUC}_{\text{p.o.}}$. If we assume that the dose is completely absorbed and that elimination is due only to hepatic metabolism, then the intrinsic hepatic clearance (Cl_{int}) is related to the oral clearance by Equation C where f_B is the free fraction in blood.

$$Cl_{\text{p.o.}} = f_B * Cl_{\text{int}} \quad \text{(C)}$$

RESULTS

The concentration of NDMA in whole blood following an i.v. bolus dose decreased biphasically (Fig. 1). The half-life of the distributive phase was 18 ± 4 min in dogs given 0.5 mg/kg, and the elimination half-life was 72 ± 8 min. The values from dogs given 1.0 mg/kg were very similar (Table 1). The AUCs for the two doses used were roughly proportional to the dose indicating that the pharmacokinetics of NDMA was linear (first order) in this dose range. The systemic clearance from blood ranged from 33.9 to 52.9 ml/min/kg. All of these clearance values approach or exceed hepatic blood flow in the dog (~40 ml/min/kg) (32) suggesting that the liver is not the only organ involved in the clearance of NDMA. The volume of distribution at steady state was about 2.0 liters/kg. The mean residence time, which represents the time needed for 63.2% of the dose to be eliminated, is a "half-life-like" term that is not susceptible to the vagaries of curve fitting. The values for the mean residence time in these dogs showed remarkably low variability (Table 1).

The dogs given the 0.5-mg/kg dose were housed in metabolism cages and urine was collected for 24 h. No NDMA was detected in the urine suggesting that the systemic clearance of NDMA was totally metabolic and that the renal clearance of NDMA was essentially zero. Since NDMA may have been reabsorbed from the urinary bladder, the true renal clearance may be greater than zero. Practically, however, the renal clearance is negligible.

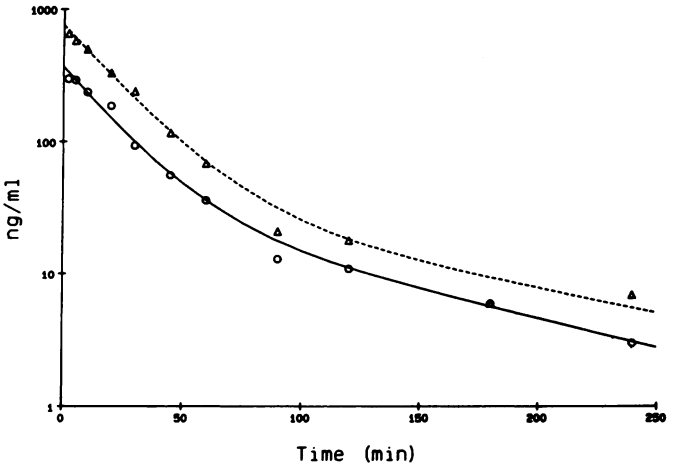

Fig. 1. Blood concentration *versus* time profile for dog 3, given 0.5 mg/kg (○) and 1.0 mg/kg (△) NDMA i.v.

Table 1 *Pharmacokinetic parameters for N-nitrosodimethylamine administered i.v. to beagles*

Dog	AUC (min•ng/ml)	Cl_s (ml/min/kg)	V_{ss} (liters/kg)	MRT (min)	$t_{½}\alpha$ (min)	$t_{½}\beta$ (min)
			0.5 mg/kg			
1	9,284	53.9	2.3	43	17	61
2	10,508	47.6	2.1	45	22	75
3	10,370	48.2	2.2	45	14	70
4	13,362	37.4	1.7	45	20	81
Mean	10,881	46.8	2.1	45	18	72
SD	1,742	6.9	0.3	1	4	9
			1.0 mg/kg			
1	26,366	37.9	1.8	47	25	41
2	25,561	39.1	1.4	37	17	49
3	20,843	48.0	2.2	45	15	81
4	29,461	33.9	1.5	45	19	124
Mean	25,558	39.7	1.7	44	19	74
SD	3,565	5.9	0.3	4	4	38

The pharmacokinetics following 1.0- and 5.0-mg/kg doses administered p.o. were studied as well. A typical blood concentration-time curve (Fig. 2), and the individual pharmacokinetic parameters (Table 2) illustrate some important points. Unlike the dose range used i.v., the pharmacokinetics of the 5.0-mg/kg p.o. dose was clearly nonlinear. The blood concentration-time curves did not decline in parallel, and the curve for the higher dose showed signs of saturated metabolism (Fig. 2). Consistent with this were the observations that the AUC and C_{max} values were not proportional to dose. The pharmacokinetics of the 1.0-mg/kg dose was probably first order since that same dose administered i.v. was first order, and the concentrations of NDMA in blood following 1.0 mg/kg p.o. did not exceed those observed after i.v. dosing. Therefore, the pharmacokinetic parameters calculated for the 1.0-mg/kg dose are valid.

The oral clearance can be considered to be a good approximation of the intrinsic hepatic clearance if certain assumptions are made. These assumptions are that the dose is totally ab-

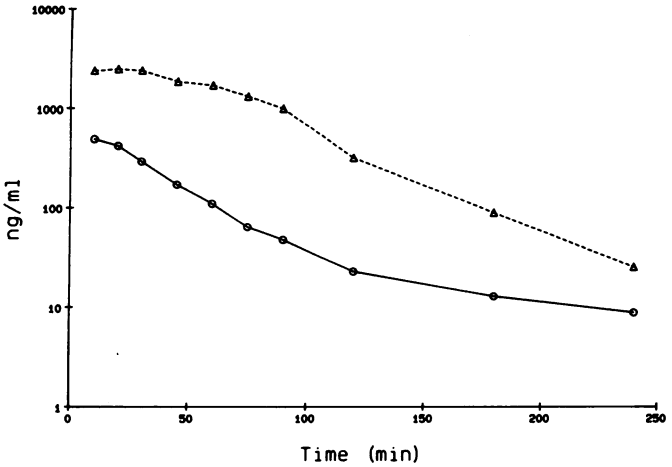

Fig. 2. Blood concentration *versus* time profile for dog 3, given 1.0 mg/kg (○) and 5.0 mg/kg (△) NDMA p.o.

Table 2 *Pharmacokinetic parameters for N-nitrosodimethylamine administered p.o. to beagles*

Dog	AUC (min•ng/ml)	C_{max} (ng/ml)	t_{max} (min)	$t_{½}$ (min)	$Cl_{p.o.}$ (ml/min/kg)	MRT (min)	F^a (%)
			1.0 mg/kg				
1	15,176	193	30	43	65.9	70	58
2	29,934	501	20	46	33.4	56	117
3	21,924	488	10	66	45.6	57	105
4	27,065	512	20	51	36.9	61	92
Mean	23,525	424	20	52	45.5	61	93
SD	6,478	154	8	10	14.6	6	26
			5.0 mg/kg				
1	224,183	2,434	20	34	22.3	67	170
2	236,686	2,896	30	36	21.1	68	185
3	196,678	2,455	20	40	25.4	58	189
4	221,768	2,923	30	45	22.5	65	151
Mean	219,829	2,677	25	39	22.8	65	174
SD	16,761	269	6	5	1.8	5	17

[a] Bioavailability calculated relative to the 1.0-mg/kg i.v. dose.

sorbed, that the free fraction of the compound in blood is 1, and that the liver is the only site of metabolism of the compound. Since the bioavailability is close to 100%, it is apparent that NDMA is completely absorbed from the gastrointestinal tract. The plasma protein binding of [^{14}C]NDMA, measured over an initial concentration range of 1 to 1000 ng/ml, was zero, and the blood/plasma concentration ratio of [^{14}C]NDMA was approximately 1 (data not shown). Therefore, it is reasonable to assume that the free fraction in blood equals 1. The $Cl_{p.o.}$ of NDMA in dogs is approximately 45 ml/min/kg.

The absorption half-life of NDMA can be estimated from the difference between the p.o. and i.v. mean residence times (31). Using this method, the absorption half-life for NDMA was 12 min.

The bioavailability of NDMA, which is the fraction of a p.o. dose that reaches the systemic circulation, was remarkably high and variable in the dogs ranging from 58 to 117%.

DISCUSSION

In the present study the pharmacokinetics of NDMA in dogs has been studied, and the most striking result is the observation that, in spite of a high systemic clearance, the oral bioavailability of NDMA is high. There are several explanations for this anomalous result that can be considered. (*a*) NDMA may be unstable in blood. This can be ruled out because NDMA added to blood *in vitro* and in actual biological samples incubated at 37°C was found to be very stable (data not shown). (*b*) At the doses used the first pass metabolism of NDMA was saturated. This is certainly true for the 5.0-mg/kg dose, but the 1.0-mg/kg dose appears to follow first order kinetics, the pharmacokinetics of 1.0 mg/kg administered i.v. are first order, and the concentrations of NDMA in blood following p.o. administration of 1.0 mg/kg were not higher than after the same dose i.v. (*c*) Since this was a crossover study and the dogs were given the i.v. doses first, significant liver damage occurred which decreased the hepatic clearance of the subsequent p.o. doses. This does not appear to be the case because activities of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, γ-glutamyl transferase in serum, and total protein concentration were within normal limits throughout the experiment except for a mild elevation of alanine aminotransferase and alkaline phosphatase in dog 3 after the first i.v. dose and before the second i.v. dose. (*d*) The most likely explanation is that a substantial fraction of the i.v. dose was metabolized in a single pass through the lung resulting in a pulmonary first pass effect. This would tend to lower the AUC after i.v. dosing and inflate the bioavailability values. It must be remembered that the lungs receive all of cardiac output so that a modest extraction ratio in the lung can contribute significantly to the clearance.

The fact still remains that the fraction of a p.o. dose reaching the general circulation is probably much greater in the dog than in the rat. In rats the bioavailability of NDMA is about 10% (12). This fact has been used to help explain the observation that chronic administration of low doses of NDMA results primarily in liver tumors whereas single high doses result in a high yield of renal tumors (1). The rationale is that at the low doses very little of the NDMA passes through the liver, but at the high doses first pass metabolism is saturated and a larger fraction of the dose reaches the systemic circulation. This concept is supported by measurements of DNA alkylation in kidney and liver following various doses of NDMA (18, 20). Given that a large fraction of a p.o. dose in dogs does pass through the liver, the organ specificity could be expected to be quite different than in the rat. The carcinogenicity of NDMA has not been tested in dogs, but chronic p.o. administration of *N*-nitrosodiethylamine resulted primarily in liver tumors (33). Since little is known about the metabolism of NDMA or *N*-nitrosodiethylamine in dogs, it is possible that the relative metabolic activities of different organs in the dog and perhaps the relative contribution of activation *versus* detoxification pathways in the dog differ substantially from those in the rat.

An interspecies comparison of the pharmacokinetics of NDMA would be interesting, but at this time adequate pharmacokinetic data are available only for rats (12), and now dogs. Some data are available for rabbits as well (13). Although the data are limited some interesting comparisons can be made. The absorption of NDMA from the gastrointestinal tract is rapid in both rats and dogs even though it has been demonstrated in rats that very little absorption occurs in the stomach (20). This can be explained from data on gastric emptying in fasted rats. In fasted rats the half-life for gastric emptying following administration of a liquid is about 12 min (34). The half-life increases to 2 h if digestible solid is given and to 4 h if undigestible solid is administered. Therefore, a dose of NDMA given by gavage to a fasted rat would enter the small intestine and be absorbed rapidly. The best available data on gastric emptying in dogs comes from studies in which radiolabeled

liver homogenate was given to the dogs (35). In 15 min 70% of the radiolabel remained in the stomach, and this decreased to 50% in 30 min and to 15% at 60 min. The behavior of a liquid should be very similar to that of the liver homogenate. The absorption half-life of NDMA in this study was about 12 min which is somewhat faster than the gastric emptying. Perhaps the absorption of NDMA from dog stomach is more rapid than rat stomach. This would not be terribly surprising since the anatomy of rat stomach is quite different than that of the dog.

The systemic clearance from blood in the dog was 47 ml/min/kg (at the 0.5-mg/kg i.v. dose). In the rat, Mico *et al.* (12) reported the systemic clearance 39 ml/min/kg. If one assumes that the entire dose of NDMA is absorbed from the gastrointestinal tract, that all of the metabolism takes place in the liver, and that all of the NDMA in blood is free (not bound to protein or cells), then the oral clearance is equal to the intrinsic hepatic clearance. In the dog it is possible that a portion of the clearance may be extrahepatic. If for the purpose of discussion this is ignored, then the oral clearance is 46 ml/min/kg in the dog and 467 ml/min/kg in the rat. Thus, despite a 10-fold difference in intrinsic clearance, the total systemic clearance in rats and dogs is similar.

The volume of distribution at steady state is also quite different in dogs and rats. In rats the V_{ss} is about 0.3 liter/kg and in dogs it is about 2.0 liters/kg. In most interspecies comparisons the volume of distribution of a compound remains relatively constant, and since NDMA is not bound to plasma protein and distributes evenly in animals it is surprising that the values are different.

These comparisons demonstrate one of the difficulties in extrapolation of data from one species to another; *i.e.*, the overall pharmacokinetics can be quite different. In extrapolating carcinogenicity data, other considerations such as the contribution of activation and detoxification pathways to the overall clearance of the compound; the status of repair processes, especially DNA repair; and the inherent susceptibility of the tissues to carcinogenesis are all confounding factors.

If humans are exposed to NDMA through environmental exposure (not by deliberate poisoning) the dose will be exceedingly low. Based on rat data, it would be expected that a very small fraction of the dose would pass through the liver. The ability of human liver to repair lesions such as O^6-methylguanine should be sufficient to substantially reduce the risk of liver cancer from this type of exposure (36). However, if it is generally true that in higher species the bioavailability of NDMA is high, then other organs will be exposed to high concentration of the carcinogen. There is evidence that other human tissues can metabolize NDMA (5–9), and repair of DNA lesions such as O^6-methylguanine does occur in tissues other than the liver (37, 38), but the rate of metabolism and rate of repair processes are not characterized well enough to even speculate about the risk of such exposure. Clearly, more data are needed on the pharmacokinetics of NDMA as well as other important nitrosamines such as the tobacco-specific nitrosamines in other mammalian species to develop a better understanding of species differences and to evaluate the feasibility of extrapolating pharmacokinetic data from animals to humans.

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